Effects of a Diphenyl Ether-type Herbicide, Chlornitrofen, and Its Amino Derivative on Androgen and Estrogen Receptor Activities

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Chlornitrofen (CNP) was widely used in large quantities as a herbicide in rice paddy fields in Japan during 1965–1994. Recently, there has been concern that chemicals in the environment may disrupt the endocrine function of wildlife and humans, but little is known about the effect of CNP on endocrine function. We have developed reporter gene assays for human androgen receptor (hAR) and human estrogen receptor-α (hERα) using Chinese hamster ovary cells. Using this assay method, we measured CNP and its amino derivative (CNP-amino) for hAR and hERα agonist/antagonist activities, comparing them with several well-known AR antagonists or ER agonists. We found that CNP and CNP-amino have potent antiandrogenic activities as well as estrogenic activities. The order of their antiandrogenic activity was CNP > vinclozolin > *o,p′*-DDT = *p,p′*-DDE > CNP-amino, and the order of their estrogenic activity was *o,p′*-DDT > CNP-amino > *p,p′*-DDT > CNP. We investigated the binding ability of CNP and CNP-amino to hAR and hERα using a receptor competitive-binding assay. The order of their binding potencies to hAR was CNP > *o,p′*-DDT = *p,p′*-DDE = CNP-amino > vinclozolin, and that of their binding potencies to hERα was *o,p′*-DDT > CNP-amino > *p,p′*-DDT = CNP. These results suggest that both CNP and CNP-amino may act as endocrine disruptors via AR and ERα in humans and other animals. Our reporter gene assays are highly sensitive and specific and are suitable for screening AR and ERα agonist/antagonists among numerous environmental chemicals. *Key words:* antiandrogenic activity, Chinese hamster ovary cells, chlornitrofen, chlornitrofen-amino, estrogenic activity, human androgen receptor, human estrogen receptor α, reporter gene assay. *Environ Health Perspect* 111:497–502 (2003). doi:10.1289/ehp.5724 available via *http://dx.doi.org/* [Online 1 November 2002]

Chlornitrofen [2,4,6-trichlorophenyl-4′-nitrophenyl ether (CNP); Figure 1] was widely used in large quantities as a herbicide to control various weeds in rice fields in Japan during the period 1965–1994. This herbicide was produced and used mostly in Japan. The amount of the active ingredient of CNP used in Japan was estimated to be 82,359 tons (Masunaga et al. 1998). Several studies reported unusually high levels of CNP residue in freshwater fish and shellfish during the application period (Ohyama et al. 1986; Watanabe et al. 1981, 1983; Yamagishi and Akiyama 1981). CNP is also known to convert to its corresponding amino derivative [2,4,6-trichlorophenyl-4′-aminophenyl ether (CNP-amino); Figure 1] by reduction of the CNP nitro group in the soil of paddy fields (Kuwatsuka 1977; Shimotori and Kuwatsuka 1978). There have also been reports of the isolation of CNP and CNP-amino from tap water and shellfish (Adachi 1994; Suzuki et al. 1983). Yamamoto et al. (1987) reported that the standardized mortality ratios of biliary tract cancer were high in Niigata prefecture, especially in the Niigata plain, and that this phenomenon could be related to the use of CNP. Thus, the use of CNP is thought to cause water pollution in rice-growing areas in Japan and lead to a high accumulation of CNP and CNP-amino in fish and shellfish in lakes and seas surrounding areas of rice cultivation.

Recently, it has been well documented that several chemicals from agricultural, industrial, and household sources possess endocrine-disrupting properties that are a potential threat to human and wildlife reproduction (Colborn 1995; Colborn et al. 1993; Jensen et al. 1995). The mechanism of action of these effects is considered to consist mainly of agonistic or antagonistic effects on hormone receptors. For example, it has already been reported that several pesticides or their metabolites such as vinclozolin, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p′*-DDE), fenitrothion, and procymidone are androgen receptor (AR) antagonists (Kelce et al. 1995; Ostby et al. 1999; Tamura et al. 2001; Wong et al. 1995) and that pesticides such as 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (*p,p′*-DDT) and methoxychlor are estrogen receptor (ER) agonists (Chen et al. 1997; Shelby et al. 1996). Moreover, it has been reported that some of the environmental estrogens such as 1,1,1-trichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane (*o,p′*-DDT), bisphenol A, and butyl benzyl phthalate also have antiandrogenic activitiy (Sohoni and Sumpter 1998). Although CNP and CNP-amino are thought to form methemoglobin, induce hepatic drug-metabolizing enzymes, and display mutagenicity (Hanioka et al. 1995; Miyauchi et al. 1981, 1983; Oguri et al. 1995), the endocrine-disrupting effects of CNP or CNP-amino have not yet been described.

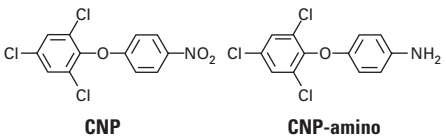

Figure 1. Chemical structures of CNP and CNP-amino.

The reporter gene assay has been widely used as an *in vitro* method for clarifying the ligand–receptor interaction by receptor agonists and antagonists. To detect the (anti)hormonal activities of environmental chemicals, some investigators have performed reporter gene assays using yeast cells, HepG2 cells, Hela cells, and so forth (Gaido et al. 1997; Maness et al. 1998; Nishikawa et al. 1999; Saito et al. 2000). However, these assays all encounter problems in the membrane transport of chemicals, sensitivity, or complicated procedures. In this study, we established two transient reporter gene assays for detecting transcriptional activities via AR and ER activities using transfection reagent FuGene6 and Chinese hamster ovary (CHO) cells based on the method of Vinggaard et al. (1999). The method is rapid, sensitive, and reproducible. Using this assay, we investigated the effects of CNP and CNP-amino on androgenic and estrogenic activities. In addition, the binding affinities of CNP and CNP-amino to human androgen receptor (hAR) and human estrogen receptor-α (hERα) were also investigated using a receptor competitive-binding assay (Satoh et al. 2000, 2001). Here we provide the first evidence that CNP and CNP-amino might be endocrine-disrupting chemicals with both antiandrogenic and estrogenic activities that act via hormone receptors.

Materials and Methods

Chemicals and cell culture materials. 5α-Dihydrotestosterone (DHT, 95% pure), testosterone (> 97% pure), 17β-estradiol (E_2, > 97% pure), estrone (98% pure), progesterone (98% pure), cortisol (> 97% pure), and dimethylsulfoxide (DMSO), used for confirming the specificity of the assay system,

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